

10/519452

PCT/DK 03/00455

10 Rec'd PCT/PTG

22 DEC 2004

REC'D 30 JUL 2003

WIPO

PCT



Kongeriget Danmark

Patent application No.: PA 2002 01021

Date of filing: 01 July 2002

Applicant: Novozymes A/S
(Name and address) Krogshøjvej 36
DK-2880 Bagsværd
Denmark

Title: Mpg added to fermentation

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)



Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

18 July 2003

Pia Høybye-Olsen



PATENT- OG VAREMÆRKESTYRELSEN

Modtaget

- 1 JULI 2002

MPG ADDED TO FERMENTATION**PVS****TECHNICAL FIELD**

5 The present invention relates to a method of increasing solubility of a polypeptide of interest during fermentation.

BACKGROUND ART

Formation of polypeptide crystals/amorphous precipitate during fermentation is
10 today seen frequently because the fermentation yields are getting higher and higher due to optimization of the fermentation recipes and/or due to identification/development or construction of more efficient production organisms.

In such cases, the polypeptides are fermented in yields that are above their solubility limit, meaning that they may be present in the culture broth in a partly
15 precipitated form. The precipitate may be in the form of crystals or as amorphous precipitates.

This causes problems in recovery where special measures have to be taken to solubilize the crystals/amorphous precipitate before removing the cells and other solids from the culture broth. These measures often result in yield losses.

20 The purpose of this invention is therefore to provide a simple and efficient solution to the above described problem.

SUMMARY OF THE INVENTION

It has surprisingly been found that the polypeptide of interest can be prevented
25 from crystallizing or precipitating by adding a carbohydrate and/or a polyol and/or a derivative thereof and/or a polymer to the culture medium before and/or during fermentation, wherein the microorganism is not, or only to a low extent, able to metabolize said carbohydrate and/or said polyol and/or said derivative thereof; in particular the present invention deals with a method for fermenting a microorganism, producing a
30 polypeptide of interest, in a culture medium of at least 50 litres, comprising:
adding one or more compounds selected from the group consisting of monopropylene glycol, ethylene glycol, trehalose, xylitol, arabitol, dulcitol, mannitol, erythritol, and sorbitol, to the culture medium before and/or during fermentation.

DETAILED DISCLOSURE OF THE INVENTION

The present invention deals with a new and surprisingly effective way of preventing the polypeptide of interest to crystallize or precipitate during the fermentation.

5 We have surprisingly found that if small amounts of, e.g., 5 % w/w of monopropylene glycol (MPG) is present during the fermentation, the formation of crystals or amorphous precipitate can be avoided, significantly delayed or significantly reduced. The MPG is only a very poor carbon source for most microorganisms or is very poorly metabolized by most microorganisms, or not metabolized at all, so it can be added before
10 starting the fermentation and/or added during the fermentation without affecting the cell growth and productivity of the peptide of interest significantly.

By avoiding formation of polypeptide crystals/amorphous precipitate during fermentation, a much more simple recovery process can be used resulting in higher yields.

15

Microorganisms

The microorganism (the microbial strain) according to the invention may be obtained from microorganisms of any genus.

In a preferred embodiment, the polypeptide of interest may be obtained from a
20 bacterial or a fungal source.

For example, the polypeptide of interest may be obtained from a gram positive bacterium such as a *Bacillus* strain, e.g., *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*,
25 or *Bacillus thuringiensis*; or a *Streptomyces* strain, e.g., *Streptomyces lividans* or *Streptomyces murinus*; or from a gram negative bacterium, e.g., *E. coli* or *Pseudomonas* sp.

The polypeptide of interest may be obtained from a fungal source, e.g. from a yeast strain such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*,
30 *Schizosaccharomyces*, or *Yarrowia* strain, e.g., *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* strain.

The polypeptide of interest may be obtained from a filamentous fungal strain such

as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* strain, in particular the polypeptide of interest
 5 may be obtained from an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium*
 10 *sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* strain.

15 Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

20 For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide of interest is produced by the source or by a cell in which a gene from the source has been inserted.

Modification of the microorganism of interest

25 The microorganism used according to the present invention may be modified in such a way that it is not, or only to a low extent, able to metabolize the chosen carbohydrate and/or polyol and/or derivative thereof; e.g., the original microorganism is able to metabolize glycerol or cyclodextrin but the modified microorganism is not, or only to a low extent.

30

Polypeptide of interest

The polypeptide of interest may be a peptide or an enzyme.

A preferred peptide according to this invention contains from 5 to 100 amino acids;

preferably from 10 to 80 amino acids; more preferably from 15 to 60 amino acids; even more preferably from 15 to 40 amino acids.

In a preferred embodiment, the method is applied to enzymes, in particular to hydrolases (class EC 3 according to Enzyme Nomenclature; Recommendations of the
5 Nomenclature Committee of the International Union of Biochemistry).

In a particular preferred embodiment the following hydrolases are preferred:

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be an acid protease, a serine protease or a metallo protease,
10 preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

15 Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Preferred commercially available protease enzymes include Alcalase™,
20 Savinase™, Primase™, Duralase™, Esperase™, Relase™, and Kannase™ (Novozymes A/S), Maxatase™, Maxacal™, Maxapem™, Properase™, Purafect™, Purafect OxP™, FN2™, and FN3™ (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases
25 from *Humicola* (synonym *Thermomyces*), e.g. from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g. from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis*
30 (WO 96/12012), a *Bacillus* lipase, e.g. from *B. subtilis* (Dartois et al. (1993), Biochimica et Biophysica Acta, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include LipolaseTM, Lipolase s UltraTM, and LipexTM (Novozymes A/S).

Amylases: Suitable amylases (α and/or β) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, α -amylases obtained from *Bacillus*, e.g. a special strain of *B. licheniformis*, described in more detail in GB 1,296,839.

10 Examples of useful amylases are the variants described in WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424, especially the variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 181, 188, 190, 197, 202, 208, 209, 243, 264, 304, 305, 391, 408, and 444.

Commercially available amylases are DuramylTM, TermamylTM, FungamylTM,
15 NatalaseTM, Termamyl LCTM, Termamyl SCTM, and BANTM (Novozymes A/S), RapidaseTM and PurastarTM (from Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus*, *Pseudomonas*, *Humicola*, *Fusarium*, *Thielavia*,
20 *Acremonium*, e.g. the fungal cellulases produced from *Humicola insolens*, *Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP
25 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include CelluzymeTM, and CarezymeTM (Novozymes A/S), ClazinaseTM, and Puradax HATM (Genencor International Inc.), and
30 KAC-500(B)TM (Kao Corporation).

Oxidoreductases

Oxidoreductases that may be treated according to the invention include peroxidases, and oxidases such as laccases, and catalases.

Other preferred hydrolases are carbohydrases including Mannaway™. Other preferred enzymes are transferases, lyases, isomerases, and ligases.

Fermentations

5 The present invention may be useful for any fermentation in industrial scale, e.g. for any fermentation having culture media of at least 50 litres, preferably at least 100 litres, more preferably at least 500 litres, even more preferably at least 1000 litres, in particular at least 5000 litres.

10 The microbial strain may be fermented by any method known in the art. The fermentation medium may be a complex medium comprising complex nitrogen and/or carbon sources, such as soybean meal, soy protein, soy protein hydrolysate, cotton seed meal, corn steep liquor, yeast extract, casein, casein hydrolysate, potato protein, potato protein hydrolysate, molasses, and the like. The fermentation medium may be a chemically defined media, e.g. as defined in WO 98/37179.

15 The fermentation may be performed as a batch, a fed-batch, a repeated fed-batch or a continuous fermentation process.

20 In a fed-batch process, either none or part of the compounds comprising one or more of the structural and/or catalytic elements is added to the medium before the start of the fermentation and either all or the remaining part, respectively, of the compounds comprising one or more of the structural and/or catalytic elements is fed during the fermentation process. The compounds which are selected for feeding can be fed together or separate from each other to the fermentation process.

25 In a repeated fed-batch or a continuous fermentation process, the complete start medium is additionally fed during fermentation. The start medium can be fed together with or separate from the structural element feed(s). In a repeated fed-batch process, part of the fermentation broth comprising the biomass is removed at regular time intervals, whereas in a continuous process, the removal of part of the fermentation broth occurs continuously. The fermentation process is thereby replenished with a portion of fresh medium corresponding to the amount of withdrawn fermentation broth.

30 In a preferred embodiment of the invention, a fed-batch, a repeated fed-batch process or a continuous fermentation process is preferred.

Carbohydrates

Slowly metabolizable carbohydrates such as pullulan, limit dextrin, and trehalose may be used according to the present invention.

In a particular embodiment of the invention the slowly metabolizable carbohydrate is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of least 0.5% (w/w). The slowly metabolizable carbohydrate is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

Polyols

A very useful subgroup of carbohydrates, polyols, may be used according to the invention. Any polyol may be used. However, a polyol selected from the group consisting of monopropylene glycol, glycerol, ethylene glycol, xylitol, arabitol, dulcitol, mannitol, erythritol, and sorbitol, is preferred.

It is to be noted that some polyols, e.g. glycerol, are rather easily metabolized by the cells, but the uptake of e.g. glycerol can be blocked, meaning that glycerol may be used according to the present invention.

In a particular embodiment of the invention the polyol is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of least 0.5% (w/w). The polyol is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

In some cases it may be an advantage to use a mixture of two or more polyols, e.g. glycerol and monopropylene glycol, or a mixture of a polyol and a slowly metabolizable carbohydrate.

Derivatives

Another very useful subgroup of carbohydrates, derivatives, may be used according to the invention. Derivatives that may be used include maillard products, methyl glycosides, glucuronic acids, amino sugars, or N-acetyl glucosamines.

In a particular embodiment of the invention the derivative is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of least 0.5% (w/w). The derivative is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

Polymers

Polymers such as polyethers (e.g. polyethylene glycol 200, polyethylene glycol 400) or their derivatives including block polymers or block copolymers of polyethylene oxide and polypropylene oxide, wherein the ends of the polymers may further be protected by an acyl group or an alkyl group, may also be used according to the present invention.

In a particular embodiment of the invention the polymer is added to the culture medium either prior to inoculation or after inoculation at an amount of at least 0.1 % (w/w); in particular at an amount of least 0.5% (w/w). The polymer is added to the culture medium either prior to inoculation or after inoculation at an amount of up to 10% w/w; preferably at an amount of up to 8% w/w; more preferably at an amount of up to 6% w/w; more preferably at an amount of up to 5% w/w; more preferably at an amount of up to 4% w/w; more preferably at an amount of up to 3% w/w; more preferably at an amount of up to 2% w/w; even more preferably at an amount of up to 1% w/w.

Extent of metabolization

The following test may be used to check whether a microorganism, producing a polypeptide of interest, is not, or only to a low extent, able to metabolize a given compound:

A suitable media for the growth of the microorganism of interest is chosen.

The media is characterized by the following parameters:

- a: The media contains glucose as the only carbohydrate source.
- b. When glucose is removed the media should only be able to support growth of a significantly lower biomass (less than 50%).
- 5 The growth of the microorganism of interest is then compared in the following 3 media:
- I: Normal media (with glucose as the only carbohydrate source)
- II: Media I without glucose
- III: Media I without glucose, but with the same C-mol of the compound to be tested.
- 10 The growth is then followed for a period of 8 hr in the 3 above mentioned media.
- Inoculation is done with a concentration of biomass that will secure that the normal media is outgrown in 75% of the time frame. The amount of biomass (OD) obtained in the different media is measured.
- The compound to be tested is defined as low metabolizable, if
- 15 $(OD_{III}-OD_{II})/(OD_I-OD_{II}) < 25\%$; preferably
- $(OD_{III}-OD_{II})/(OD_I-OD_{II}) < 20\%$; more preferably
- $(OD_{III}-OD_{II})/(OD_I-OD_{II}) < 15\%$; more preferably
- $(OD_{III}-OD_{II})/(OD_I-OD_{II}) < 10\%$; more preferably
- $(OD_{III}-OD_{II})/(OD_I-OD_{II}) < 5\%$; more preferably
- 20 $(OD_{III}-OD_{II})/(OD_I-OD_{II}) \leq 0\%$.
- In Example 1 various compounds are tested according to this test.

Recovery of the polypeptide of interest

- A further aspect of the invention concerns the downstream processing of the
- 25 fermentation broth. After the fermentation process is ended, the polypeptide of interest may be recovered from the fermentation broth, using standard technology developed for the polypeptide of interest. The relevant downstream processing technology to be applied depends on the nature of the polypeptide of interest.

- A process for the recovery of a polypeptide of interest from a fermentation broth
- 30 will typically (but is not limited to) involve some or all of the following steps:

- 1) pre-treatment of broth
- 2) removal of cells and other solid material from broth (primary separation)
- 3) filtration

- 4) concentration
- 5) filtration
- 6) stabilization and standardization.

Apart from the unit operations listed above, a number of other recovery
 5 procedures and steps may be applied, e.g., pH-adjustments, variation in temperature,
 treatment of enzyme solution with active carbon, and use of various adsorbents.

10 The invention is further illustrated in the following examples, which are not
 intended to be in any way limiting to the scope of the invention as claimed.

Example 1

15 Evaluation of the suitability of different polyols and carbohydrates as carbon-sources for
 micro-organisms.

Shake flask media

20 **Med-F 18** shake flask medium (concentrations are after final mixing).

Part A: Bacto-peptone 0.5; Yeast Extract 0.5 g/l; Magnesium sulphate 7 H₂O 0.5 g/l;
 Ammonium sulphate 2 g/l; Calcium chloride 2H₂O 0.1 g/l; Citric acid 50 mg/l; trace metals
 (MnSO₄ H₂O 2.5 mg/l; FeSO₄ 7H₂O 9.9 mg/l; CuSO₄ 5H₂O 1.0 mg/l; ZnCl₂ 1.0 mg/l);
 Pluronic 0.1 g/l; pH adjusted to 6.7

25 Part B: 5 g/l Potassiumdihydrogenphosphate pH adjusted to 6.7 with NaOH.

Part C: carbon source equivalent to 0.08 mol carbon per L (e.g. 2.5 g/L glucose).

Demineralized water is used for the preparation of all media.

After sterilization for 20 minutes at 121°C part A, B and C is mixed.

30

Strain: *Bacillus licheniformis*

Procedure for shake flask evaluation of suitability of different polyols and carbohydrates
 as alternative carbon-sources:

First the cells were grown in a pre-culture that secured good growing cells.

Each shake flask is then inoculated with the same amount of cells based on the OD_{650nm} measurement.

- 5 An inoculum strength of $OD \times ml \text{ cell suspension} = 80$ was used in this case. Resulting in an $OD=0.8$ in the shake flask at time 0.

Three shake flasks of each type were inoculated.

Shake flask types:

- 10 I: Med-F 18 with the normal part C added, where C is glucose (resulting in a medium with 2.5 g/l of glucose (equivalent to 0.08 C-mol per Liter).

II: Med-F 18 without part C, resulting in a medium without any glucose

- 15 III: Med-F 18 with part C replaced by containing one of the following:

- 2.1 g/l MPG (equivalent to 0.08 C-mol per Liter)
- 2.1 g/l PEG 200 (equivalent to 0.08 C-mol per Liter) or
- 2.4 g/l Sucrose (equivalent to 0.08 C-mol per Liter)

Resulting in media with 2.5 g/l MPG; 2.5g/l PEG 200 or 2.5 g/l Sucrose, respectively.

20

By an error the media was prepared with 2.5 g/l MPG; 2.5 g/l PEG 200; and 2.5 g/l sucrose. This should therefore result in a lightly higher OD in these media compared to the intended media, if the compounds were easily metabolized.

- 25 The shake flasks were then incubated at $37^{\circ}C$ at 300 rpm in 8 hr.

The OD_{650nm} was measured at 6 and 8 hr. and the following results were obtained:

Test of MPG:

Average OD_{650nm} found for the tree shake flask at time 6 and 8 hr.

$OD_{650 \text{ nm}}$ Time [hr.]	OD_I	OD_{II}	OD_{III}	$\frac{(OD_{III}-D_{II})}{(OD_I-OD_{II})}$
6	4.23	1.51	1.61	3.8%
8	4.08	1.46	1.57	4.3%

From the results it is clear that MPG is only very slowly or not at all metabolized by the strain used in this example. It is also clear that the culture is fully outgrown after 6 hr. as the OD is not increased in medium I going from 6 to 8 hr.

5

Test of PEG 200:

Average OD_{650nm} found for the three shake flask at time 6 and 8 hr.

OD _{650 nm} Time [hr.]	OD _I	OD _{II}	OD _{III}	$\frac{(OD_{III}-D_{II})}{(OD_I-OD_{II})}$
6	4.23	1.51	1.50	-0.2%
8	4.08	1.46	1.45	-0.4%

From the results it is clear that PEG 200 only very slowly or not at all metabolized by the strain used in this example.

10

Test of sucrose:

Average OD_{650nm} found for the three shake flask at time 6 and 8 hr.

OD _{650 nm} Time [hr.]	OD _I	OD _{II}	OD _{III}	$\frac{(OD_{III}-D_{II})}{(OD_I-OD_{II})}$
6	4.23	1.51	4.23	100.1%
8	4.08	1.46	4.02	97.7%

From the results it is clear that Sucrose is easy metabolized by the strain used in this example.

15

Example 2

Increased enzyme solubility in fermentation broth by addition of MPG to the fermentation

process

20

Materials and methods:

Strain: *Bacillus licheniformis*

Polypeptide of interest: α -amylase

25

Media:

In all cases unless otherwise describe tap water was used. All media were sterilized by methods known within the art to ensure that the fermentations were run as mono-cultures.

5

First inoculum medium:

LB agar: 10 g/l peptone from casein; 5 g/l yeast extract; 10 g/l Sodium Chloride; 12 g/l Bacto-agar adjusted to pH 6.8 to 7.2. Premix from Merck was used.

10 **Transfer buffer:**

M-9 buffer (deionized water is used): Di-Sodiumhydrogenphosphate 2 H₂O 8.8 g/l; Potassiumdihydrogenphosphate 3 g/l; Sodium Chloride 4 g/l; Magnesium sulphate 7 H₂O 0,2 g/l.

15 **Inoculum shake flask medium (concentration is before inoculation):**

PRK-50: 110 g/l soy grits; Di-Sodiumhydrogenphosphate 2 H₂O 5 g/l; pH adjusted to 8.0 with NaOH/H₃PO₄ before sterilization.

Make-up medium (concentration is before inoculation):

- 20 Tryptone (Casein hydrolysate from Difco) 30 g/l; Magnesium sulphate 7 H₂O 4 g/l; DiPotassiumhydrogenphosphate 7 g/l; Di-Sodiumhydrogenphosphate 2 H₂O 7 g/l; DiAmmoniumsulphate 4 g/l; Citric acid 0.78 g/l; Viatmins (Thiamin-dichlorid 34.2 mg/l; Riboflavin 2.9 mg/l; Nicotinsyre 23 mg/l; Calcium D-pantothenat 28.5 mg/l; Pyridoxal-HCl 5.7 mg/l; D-biotin 1.1 mg/l; Folin acid 2.9 mg/l); Trace metals (MnSO₄ H₂O 39,2 mg/l; 25 FeSO₄ 7H₂O 157 mg/l; CuSO₄ 5H₂O 15.6 mg/l; ZnCl₂ 15.6 mg/l); Antifoam (SB2121) 1.25 ml/l; pH adjusted to 6.0 with NaOH/H₃PO₄ before sterilization.

Feed medium:

Glucose, 1H₂O 820 g/l;

30

Procedure for inoculum steps:

First the strain was grown on LB agar slants 1 day at 37°C.

The agar was then washed with M-9 buffer, and the optical density at 650 nm (OD_{650 nm})

of the resulting cell suspension was measured.

The inoculum shake flask (PRK-50) is inoculated with an inoculum of $OD_{650nm} \times \text{ml cell suspension} = 0.1$.

The shake flask was incubated at 37°C at 300 rpm for 20 hr.

- 5 The fermentation in the main fermentor (fermentation tank) was started by inoculating the main fermentor with the growing culture from the shake flask. The inoculated volume was 10% of the make-up medium (80 ml for 800 ml make-up media).

Fermentor Equipment:

10

Standard lab fermentors were used equipped with a temperature control system, pH control with ammonia water and phosphoric acid, dissolved oxygen electrode to measure >20% oxygen saturation through the entire fermentation.

15 Fermentation parameters:

Temperature: 41°C

The pH was kept between 6.8 and 7.2 using ammonia water and phosphoric acid

20 Control: 6.8 (ammonia water); 7.2 phosphoric acid

Aeration: 1.5 liter/min/kg broth weight

Agitation: 1500 rpm

25

Feed strategy:

0 hr. 0.05 g/min/kg initial broth after inoculation

8 hr. 0.156 g/min/kg initial broth after inoculation

End 0.156 g/min/kg initial broth after inoculation

30

Experimental setup:

Three fermentations were run in parallel all with the same inoculation material.

Fermentation A was run as described above.

Fermentation B was run as described above but 50 g/L MPG (monopropyleneglycol) was added to the make-up medium before inoculation.

Fermentation C was run as described above but 50 g/L MPG was added to the fermentation 24 hours after inoculation.

- 5 (In fermentation B and C, the concentrations of MPG is given as the concentration based on the volume of the make-up medium before inoculation.)

Samples were taken after 3 days of fermentation. The samples were split in two identical parts (sample I and sample II). Sample II was centrifuged at 15000 g_{av} in 20 minutes at
10 38°C. The resulting supernatant was then filtered through a 0.2µm filter (Sartorius Minosart, order no.: 16534) (sample II_{sup}).

The alpha-amylase activities in sample I and sample II_{sup} was then measured by methods known within the art (for example method for alpha-amylase activity measurements described in WO 95/26397 can be used). However, when measuring samples where the
15 enzyme can be in a partly solid form, samples have to be treated with urea prior to analysis. By diluting the samples 1:50 (w/v) in a solution containing 40% (w/w) urea, 25.5 mg/L Brij 35 and 4.4 g/L CaCl₂·2H₂O both crystalline and precipitated alpha-amylase is brought into solution in an active form and it can then be further diluted in the buffer used in the activity assay.

- 20 Both sample I and sample II_{sub} are diluted in the urea-buffer as described above before being analysed.

The enzyme activity in sample II_{sup} is a measure of the soluble activity, whereas the activity in sample I is a measure of the total activity (both soluble and crystallized and precipitated activity).

25

Results:

The following activities were found after 3 days of fermentation. The total activity found in fermentation A is used set to 100% and the soluble activity (i.e. the activity in sample II_{sup})
30 is given relative to the activity in sample I.

Fermentation name	Description	Enzyme activity in total broth Sample I	Enzyme activity in solution Sample II _{sup}
Fermentation A	Standard	100 %	19 %
Fermentation B	Addition of MPG to make-up media	101 %	53 %
Fermentation C	Addition of MPG after 1 day of fermentation	99 %	95 %

From the result it is clear that addition of MPG to the media have a very significant effect on the amount of enzyme in solution in the fermentation broth.

Modtaget
- 1 JULI 2002

PVS

CLAIMS

- 5 1. A method for fermenting a microorganism, producing a polypeptide of interest, in a culture medium of at least 50 litres, comprising:
adding one or more compounds selected from the group consisting of monopropylene glycol, ethylene glycol, trehalose, xylitol, arabitol, dulcitol, mannitol, erythritol, and sorbitol, to the culture medium before and/or during fermentation.
- 10 2. The method according to claim 1, wherein the microorganism is a bacterium or a fungus.
3. The method according to claim 2, wherein the bacterium is a *Bacillus* strain.
- 15 4. The method according to claim 1, wherein the polypeptide is an enzyme or a peptide.
5. The method according to claim 4, wherein the enzyme is selected from the group consisting of a hydrolase, a protease, an amylase, a cellulase, a lipase, an
20 oxidoreductase, and a carbohydrase.
6. The method according to claim 4, wherein the peptide contains from 5 to 100 amino acids.
- 25 7. The method according to claim 1, wherein the compound is added in an amount of least 0.1 % (w/w) of the culture medium.
8. The method according to claim 1, wherein the compound is monopropylene glycol.